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Elf5 conditional knockout mice reveal its role as a master regulator in mammary alveolar development: Failure of Stat5 activation and functional differentiation in the absence of Elf5

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ABSTRACT

The transcription factor Elf5 plays an important role in mammary gland development. However, because of the embryonic lethality of Elf5 straight knockout mice, prior studies have been limited to experiments with Elf5 haploinsufficient animals, overexpression systems or transplants. Here, we have utilized K14-Cre to generate mammary-gland specific Elf5 conditional knockout mice. During pregnancy, Elf5-null mammary epithelium completely failed to initiate alveologenesis, and a characteristic of virgin ductal epithelial cells persisted postpartum. We demonstrate that the loss of Elf5 leads to the absence of alveolar secretory markers confirming previous published data. Interestingly, the developmental block due to a lack of Elf5 could not be restored by multiple gestations. Elf5-null mammary epithelial cells also display disorganized cell structures as evident by altered cell polarities, which might be the cause for collapsed lumina. We observe reduced levels of Stat5 and attenuated Stat5 activity as measured by p-Stat5 levels both in Elf5-null mammary glands as well as cultured mammary epithelial cells. This data suggests that the failure of alveolar and lactogenic differentiation due to the loss of Elf5 is mediated in part due to impaired Stat5 activity. In support of this hypothesis, we show by ChIP experiments that Stat5a promoter contains a conserved Elf5-binding site that is occupied by Elf5 in mammary glands. Mammary epithelia lacking Elf5 exhibited downregulation of several other critical genes involved in alveologenesis, suggesting Elf5 as a master regulator in alveolar development. We propose a model for Elf5-mediated alveolar development, in which Elf5 regulates the expression of key mediators of the PrlR/Jak2/Stat5 signaling pathway.

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Introduction

The generation of the mature and functional lobuloalveolar units of the mammary gland is a well-coordinated developmental program (Oakes et al., 2006). This process is governed by endocrine signals generated predominantly by prolactin and steroid hormones such as progesterone and a network of critical transcription factors that activates target genes involved in cell fate determination, proliferation and differentiation (Briskin and Rajaram, 2006). One such transcrip-

tion factor is E74-like factor 5 (Elf5, also known as ESE-2), a member of the Epithelium Specific Ets (ESE) subfamily belonging to Ets (E twenty-six)-domain transcription factor family (Sharrocks, 2001). All Ets family proteins contain a conserved DNA binding domain called Ets-domain that binds to regulatory regions of target genes (Sharrocks, 2001). As transcription factors, Ets proteins regulate a spectrum of normal biological processes including development, differentiation, homeostasis, proliferation and apoptosis (Sharrocks et al., 1997). Not surprisingly, many of the Ets family members not only contribute to normal cellular development, but have oncogenic and tumor suppressive activity and their mis-regulation is associated with many of the processes that lead to cancer initiation, progression and metastasis (Desmaze et al., 1997; Dittmer and Nordheim, 1998; Peter et al., 1997). Indeed, recent studies have shown that the loss of Elf5 is frequently associated with human mammary carcinoma cells and in breast cancer tissues suggesting a potential role of this transcription factor to act as a tumor suppressor in breast cancer (Ma et al., 2003; Zhou et al., 1998).

Elf5 has been shown to be restricted to tissues rich in glandular or secretory epithelial cells such as mammary gland, salivary gland, kidney, lung and stomach (Lapinskas et al., 2004; Metzger et al., 2007). Expression of Elf5 has also been discovered in the inner root

Abbreviations: AQP5, aquaporin 5; Cldn7, claudin 7; Elf5, E74-like factor 5; ER α , estrogen receptor alpha; ES, embryonic stem; ESE, epithelium specific Ets; Ets, E twenty-six; Id2, inhibitor of DNA binding 2; IKK α , inhibitor of kappa B kinase alpha; Jak2, janus kinase 2; K14, cytokeratin 14; K18, cytokeratin 18; Lmo4, LIM domain only 4; MMTV, mouse mammary tumor virus; Muc-1, mucin 1; NKCC1, Na–K–Cl cotransporter 1; Npt2b, Na–Pi type IIb cotransporter; PGK-neo, phosphoglycerate kinase-neomycin; PR, progesterone receptor; PrlR, prolactin receptor; SOCS, suppressor of cytokine signaling; Stat5, signal transducer and activator of transcription 5; p-Stat5, phosphorylated Stat5; TEB, terminal end buds; Wap, whey acidic protein.

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sheath of hair follicle and the differentiated keratinocytes in skin (Choi et al., 2008; Tummala and Sinha, 2006). In addition, *Elf5* is highly restricted to the extraembryonic ectoderm, which is essential for mammalian placental formation and the survival of the embryo in utero (Donnison et al., 2005). Indeed, the generation of *Elf5* knockout mice has demonstrated that embryos lacking both alleles of *Elf5* die very early during embryogenesis and exhibit specific defects in the formation of extraembryonic ectoderm (Donnison et al., 2005; Zhou et al., 2005). Significantly, *Elf5* is highly induced during pregnancy and is primarily restricted to the luminal epithelial cells of mammary gland, and coincidentally, heterozygous *Elf5* females display impaired mammary alveolar morphogenesis in specific strains of mice (Zhou et al., 2005). Furthermore ectopic expression of *Elf5* can rescue the alveolar dysmorphogenesis phenotype of mammary epithelial cells lacking prolactin receptor (*PrIR*) (Harris et al., 2006).

These data support the notion that *Elf5* is one of the important factors that regulate developmental events of mammary alveolar epithelial cells and its regulation involves the prolactin signaling pathway. However, the underlying molecular and cellular events controlled by *Elf5* are not fully understood due to the early embryonic lethality of mice lacking both *Elf5* alleles. Therefore, *in vivo* studies of *Elf5* in mammary epithelial development have been performed in *Elf5* heterozygous mice or with transplants from *Elf5* null animals (Oakes et al., 2008; Zhou et al., 2005). Although these studies have clearly established that *Elf5* is essential for lobuloalveologenesis, the limitations of the aforementioned systems warrant further investigations with mouse models that allow studies involving the complete loss of *Elf5* in a more physiological context.

In this study, we established an *Elf5* tissue-specific knockout mouse line to circumvent the embryonic lethality associated with mutation of the *Elf5* gene during embryogenesis and to examine the effect of the complete loss of *Elf5* in different stages of development. To elucidate the precise roles of *Elf5* in mammary epithelial development, we have utilized the Cre-loxP system leading to mammary epithelium-specific deletion of *Elf5*. We successfully generated mice (*K14-Cre/Elf5^{f/f}*) carrying two *Elf5* floxed alleles and a *Cre* transgene under control of the human *cytokeratin 14* (*K14*) gene promoter, which is active in dividing cells of several stratified epithelial tissues, including skin and mammary gland. Our data show that *K14-Cre/Elf5^{f/f}* dams lack *Elf5* in mammary gland tissue, and completely fail to initiate lobuloalveologenesis and maintain virgin-like ductal features with an underdeveloped morphology even at parturition. We show that the morphological alterations in *Elf5* null mammary gland are associated with alterations of critical components of the *PrIR/Jak2/Stat5* signaling pathway as well as other known mediators of the mammary gland developmental program. Taken together, our *K14-Cre*-mediated knockout of *Elf5* unequivocally demonstrates that this transcription factor is critical for the mammary gland development during alveologenesis. The development of *Elf5*-null mouse model will facilitate a better understanding of the molecular mechanisms that underlie the alveolar switch.

Materials and methods

Generation of conditional *Elf5* knockout mice

The mouse *Elf5* genomic DNA was isolated by a PCR screening from a bacterial artificial chromosome clone of a 129 strain mouse embryonic stem (ES) cell genomic library. The vector backbone for the targeting construct was PGKneoF2L2DTA, containing a PGK-neo cassette flanked by loxP and FRT sites (the plasmid was provided kindly by Dr. P. Soriano, Fred Hutchinson Cancer Research Center, Seattle). To select against ES cells that have incorporated the targeted DNA by non-homologous recombination, PGK-dta encoding the *diphtheria toxin* gene was used as a negative selectable marker. The *Elf5* targeting vector was designed to contain two homologous arms

for efficient homologous recombination (Fig. S1A). For 3' homologous arm, a 3 kb fragment with exon 7 was amplified by PfuUltra polymerase (Stratagene), introducing a *NheI* site on the 5'-end and a *HindIII* site on the 3'-end of the amplification product. The PCR product was cloned into the *NheI*–*HindIII* sites of the PGKneoF2L2DTA vector. A 5.3 kb fragment having exons 4 and 5 was also amplified to introduce a *SacII* site at the 5'-end and a *NotI* site at the 3'-end. The 5.3 kb 5' homologous arm was then cloned into the *SacII*–*NotI* sites of the PGKneoF2L2DTA vector carrying a 3 kb of the 3' homologous arm. The floxed region was amplified from a 1.2 kb *XmaI* fragment with the exon 6 containing most of *Ets* domain, and then introduced into *XmaI* site of the PGKneoF2L2DTA vector harboring two homologous arms. This cloning into *XmaI* site allowed the insertion of the floxed region between the first loxP site and the first FRT site. The final targeting vector was linearized using *SacII*, phenol–chloroform purified, and electroporated into ES cells. The selection and expansion of ES cell clones were performed by Gene Targeting and Transgenic Core at Roswell Park Cancer Institute.

Two correctly targeted clones were used to generate chimeras and mice judged to be highly chimeric on the basis of agouti coat color were crossed with C57BL/6 mice. After getting germ-line chimeras, the offspring were identified by Southern blot analysis. The Southern blot analysis using *XhoI*–*Sall* genomic digestion and 5' probe exhibited two distinct bands of 11.7 kb (floxed allele) and 9.7 kb in size (wild-type allele). The *HpaI* Southern blot with 3' probe yielded bands of 8.8 kb (floxed allele) and 6.8 kb in size (wild-type allele). Thereafter mice were intercrossed to obtain progenies and crossed with *K14*- or *MMTV-Cre* (D-line) transgenic mouse lines (The Jackson Laboratory) to generate *Cre*-mediated knockout mice of *Elf5*. Genotyping of progenies was performed by PCR analysis of tail DNA using four primers (Fig. 1A). For the wild-type allele, a 661 bp product was obtained from the combination of primer A (5'-TTCCGAGTGGT-TAGGTCAGAAGCC-3') and primer D (5'-GAGTTTCTTCTCCTGC-CACCCGTG-3'). The primer B (5'-GACAATAGCAGGCATGCTGGGGAT-3') was located within the PGK-neo cassette and generated a 424 bp product only on the floxed allele with primer D. The primer set C (5'-CCTCTCACAGTGACTGCAGTTAAGTCCG-3') and D was utilized to genotype the knockout allele (765 bp). All experimental procedures involving mice were approved by the Institutional Animal Care and Use Committee of the State University of New York at Buffalo.

Whole-mount analysis and histological analysis

For whole-mount staining, fourth mammary glands were excised and spread on microscope slides. The tissues were fixed in Carnoy's fixative [6 parts 100% ethanol, 3 parts chloroform, 1 part glacial acetic acid], washed in 70% ethanol, hydrated by passing through decreasing ethanol concentration and stained in carmine alum stain [0.2% carmine, 0.5% aluminum potassium sulfate] overnight at room temperature. After staining, the slides were dehydrated through increasing ethanol concentrations, cleared in xylene and mounted with Permount (Fisher Scientific).

For histological analysis, mammary gland specimens were fixed overnight in neutral buffered formalin [3.7% formaldehyde buffered to pH 6.8–7.2 with monobasic and dibasic sodium phosphate], dehydrated and embedded in paraffin. Tissue blocks were sectioned into 5 μ m and stained with hematoxylin and eosin.

Primary mammary epithelial cell culture and viral infection

Isolation and adenoviral infection of primary mammary epithelial cells were performed as described previously (Naylor et al., 2005). Briefly, primary mammary epithelial cells were infected with either Ad-Cre or Ad-GFP (Vector Biolabs) in suspension for 45 min before being plated at equal density on BM (Basement Membrane) matrix (Matrigel, BD Biosciences). Cells were cultured in DMEM/F12 medium

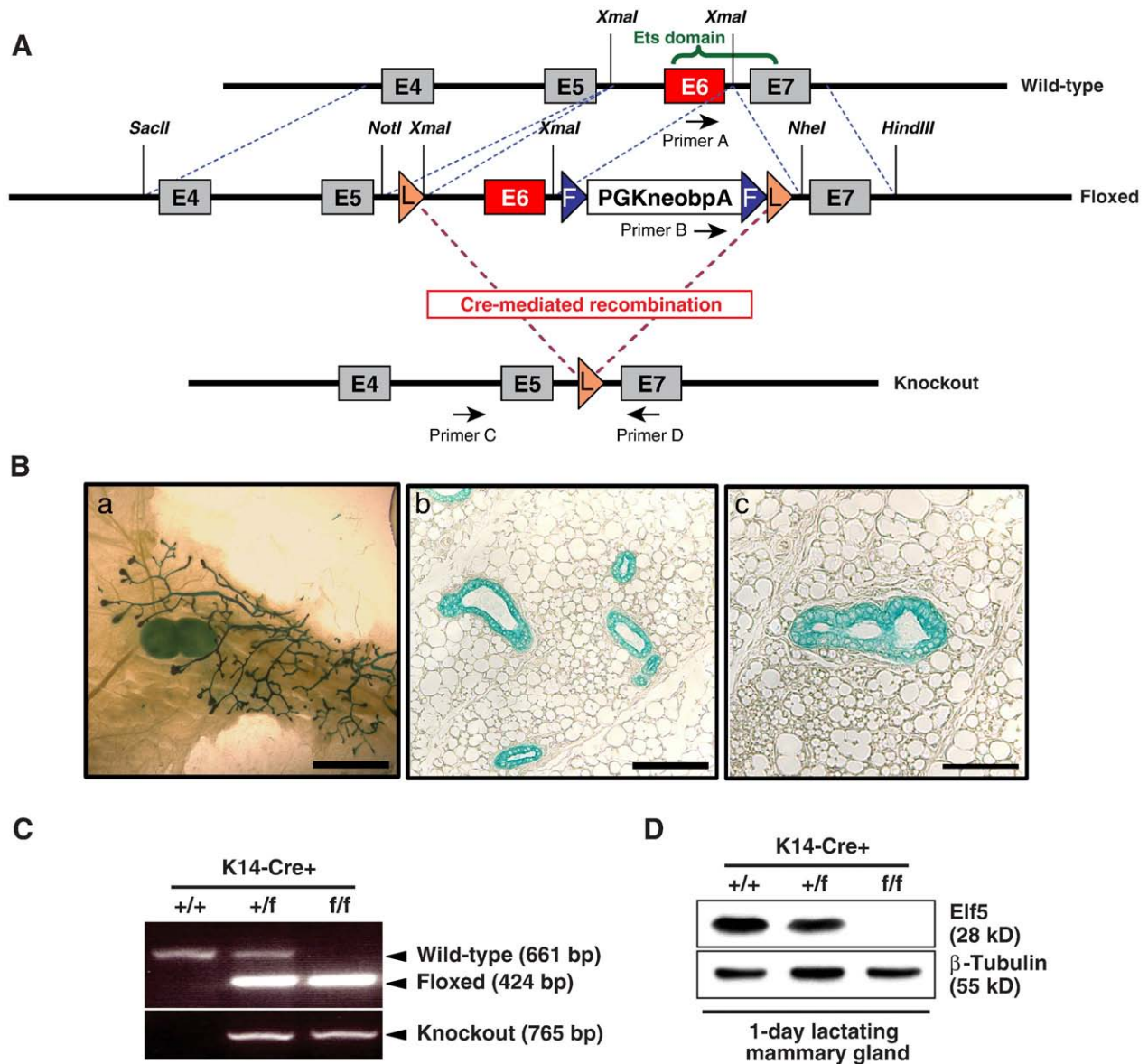


Fig. 1. Generation of *Elf5* conditional knockout mice. (A) Schematic representations of the wild-type (+) *Elf5* allele, the floxed (f) allele and the predicted *Elf5* knockout allele after Cre-mediated deletion. Red box indicates exon 6 encoding for most of the Ets domain of the *Elf5* gene; gray and white boxes indicate exons of the *Elf5* gene and a PGK-neo cassette, respectively; orange triangles flanking both the exon 6 and the FRT-flanked PGK-neo cassette display loxP sites. The positions of the primers used for genotyping are denoted by small arrows. (B) Mammary epithelial X-gal staining. To determine the efficiency of excision of K14-Cre transgene, whole-mount X-gal staining on a 5 week ROSA K14-Cre mammary gland revealed staining in both ductal network and terminal end buds (a). In cross-section, the extent of staining in the ROSA K14-Cre mice is seen to affect the majority of the luminal epithelial cells and myoepithelial cells (b). (c) Higher magnification of the mammary epithelium shows Cre activity in both mammary luminal and myoepithelial cells. (C) PCR analysis on genomic DNA of tail biopsies for genotyping of K14-Cre/*Elf5*^{+/+}, K14-Cre/*Elf5*^{+/f} and K14-Cre/*Elf5*^{f/f} animals. The K14 promoter is active in skin, accounting for the presence of the knockout allele in the tail clip. Primers A, B, C and D were used to amplify genomic DNA. While wild-type mice show a single band of 661 bp PCR product, the K14-Cre/*Elf5*^{+/f} mice display all bands of 661 bp wild-type allele (primers A/D), 424 bp floxed allele (primers B/D) and 765 bp knockout allele (primers C/D). *Elf5* null mutant mice (K14-Cre/*Elf5*^{f/f}) are identified by the absence of wild-type allele and the presence of both the floxed and the knockout alleles. (D) Western blot analysis to verify the deletion of *Elf5* protein expression in mammary glands of *Elf5* mutants. Protein was extracted from 1-day lactating mammary gland of wild-type, heterozygous and homozygous *Elf5* mutant mice with K14-Cre-mediated recombination. The lysates were subjected to Western blot analysis using anti-*Elf5* or anti- β -tubulin antibodies, as indicated. No *Elf5* protein expression was detected in K14-Cre/*Elf5*^{f/f} mammary gland. β -Tubulin protein blot serves as a loading control. No *Elf5* protein expression was detected in K14-Cre/*Elf5*^{f/f} mammary gland.

containing insulin (5 μ g/ml), Hydrocortisone (1 μ g/ml), EGF (10 ng/ml), FBS (10%), penicillin/streptomycin (100 U/ml and 100 μ g/ml respectively), Gentamycin (50 μ g/ml). For inducing differentiation, 3 μ g/ml of prolactin was added.

Immunohistochemistry and immunofluorescence

Mammary glands were fixed in neutral buffered formalin overnight and embedded in paraffin. 5 μ m sections were baked for 30 min at 60 $^{\circ}$ C, and then deparaffinized. For immunohistochemistry, heat-induced antigen retrieval was performed by microwaving sections in

10 mM sodium citrate, pH 6.0 for 20 min. Endogenous peroxidase activity was quenched by incubating the sections in 3% hydrogen peroxide solution for 10 min. All incubations were performed at room temperature unless otherwise stated. Endogenous biotin was blocked using the Streptavidin/Biotin blocking kit according to the manufacturer's instructions (Vector Laboratories). After blocking in 5% BSA/0.05% Tween-20, primary antibodies were applied (*Elf5*, 1:100, Santa Cruz Biotechnology; p-Stat5, 1:200, Cell Signaling Technology; Wap, 1:300, Santa Cruz Biotechnology; PR, 1:50, Abcam; Ki67, 1:50, Novocastra; ER α , 1:500, Santa Cruz Biotechnology; Laminin, 1:50, Sigma) and the sections were incubated overnight at 4 $^{\circ}$ C.

Subsequently, sections were incubated with biotinylated secondary antibodies [1:300] for 30 min. Visualization of the bound biotin was then performed using HRP-labeled streptavidin followed by DAB substrate kit or Streptavidin–FITC followed by TOPRO staining (for nucleus) according to manufacturer's protocol (Vector Laboratories).

The following rabbit polyclonal antibodies and dilutions were used for immunofluorescence: AQP5 at 1:100 (Alomone Labs), Npt2b at 1:50 (Alpha Diagnostic), NKCC1 at 1:1000 (gift from Dr. Jim Turner, NIH), SMA at 1:250 (Sigma), (Abcam) and Stat5a at 1:100 (Santa Cruz Biotechnology). Mouse monoclonal anti- β -catenin antibody (1:500, Sigma) was used to visualize cell membranes. Antigen retrieval was performed either in 1 mM EDTA (pH 8.0) for 15 min or in 10 mM sodium citrate (pH 6.0) for 10 min. After blocking with M.O.M. kit (Vector Laboratories), sections were incubated with primary antibodies overnight at 4 °C, followed by 45-min incubation with Alexa Fluor 568-conjugated anti-rabbit antibodies (1:750, Molecular Probes) and FITC-conjugated anti-mouse antibodies (1:500, BD Biosciences). The slides were mounted with Vectashield (Vector Laboratories), and the immunofluorescence was viewed under a Nikon microphoto-FXA microscope (Nikon) or Leica Confocal Microscope.

Protein extraction and Western blot analysis

Protein extracts from primary epithelial cell cultures were made in RIPA buffer [50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1%(v/v) Triton X-100, 1%(w/v) sodium deoxycholate, 0.1%(v/v) SDS] supplemented with complete protease inhibitor cocktail tablet (Roche) and phosphatase inhibitors (sodium orthovanadate (1 mM) and sodium fluoride (10 mM)). Mammary gland protein extracts were prepared by grinding tissue into a fine powder in liquid nitrogen and subsequently dissolving in RIPA buffer supplemented with complete protease inhibitor cocktail tablet (Roche) and phosphatase inhibitors. The lysates were centrifuged at 13,000 rpm for 15 min and the supernatant was collected as extracted protein. Protein extracts were separated by SDS-PAGE and then transferred to a PVDF (polyvinylidene difluoride) membrane (Bio-Rad). After blocking, the blots were probed with primary antibodies followed by a second incubation with secondary antibodies [1:20,000 or 1:30,000] conjugated to HRP. Primary antibodies were as follows: goat anti-Elf5 antibodies [1:1000] (Santa Cruz Biotechnology), alpha casein antibody [1:2000] (gift from Dr. Yuping Sun, Harvard University), Stat5a [1:2000] (Santa Cruz Biotechnology), p-Stat5 [1:1000] (Cell Signaling), Cre [1:2000] (Novagen). For loading control, the blots were also probed with mouse anti- β -tubulin antibodies [1:5000] (Chemicon) and anti-mouse IgG secondary antibody [1:40,000] conjugated to HRP. The chemiluminescence detection of HRP-conjugated secondary antibodies was performed using the LumiGLO Reserve Chemiluminescent Substrate kit (KPL).

RT-PCR and real-time RT-PCR analyses

Total RNAs were isolated from the primary cell cultures in BM matrix and the mouse mammary glands using TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions and then further purified by phenol/chloroform extraction. Subsequently, the purified RNA was treated with TURBO DNA-free kit (Ambion) to remove genomic DNA contamination. The treated RNA was reverse transcribed into cDNA by using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. PCR amplification was carried out using specific primer set for mouse *NKCC1* gene (Table 1). β -Actin and *HPRT* (hypoxanthine-guanine phosphoribosyl-transferase) were amplified as loading controls. As an internal negative control, an RNA aliquot that has not been subjected to reverse transcriptase treatment was also amplified to ensure that the amplified PCR product did not arise from amplification of contaminating

genomic DNA. PCR cycling was performed using the following conditions: denaturation at 94 °C for 4 min; 30 cycles of amplification with denaturation at 94 °C for 20 s, annealing at 48 °C for 30 s, extension at 72 °C for 1 min; final extension at 72 °C for 10 min. Amplified PCR products were visualized by agarose gel electrophoresis.

Real-time RT-PCR was performed on a Bio-Rad iCycler iQ Real Time PCR system (Bio-Rad Laboratories) using iQ SYBR Green Supermix (Bio-Rad Laboratories) or mouse RT² Profiler PCR Array kit for JAK/STAT signaling pathway-relevant genes (SuperArray). Sequences of gene-specific primer pairs for real-time RT-PCR analyses are listed in Table 1. 1 μ l of diluted cDNA, 12.5 μ l of 2 \times SYBR Green Supermix and specific primers in a total of 25 μ l were amplified with the following protocol: 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 8.5 min, and 40 cycles at 95 °C for 15 s/60 °C for 1 min. The specific primer sets were used at a final concentration of 0.2 μ M each. For RT² Profiler PCR Array, RT² First strand kit and RT² SYBR Green/Fluorescein qPCR master mix were used. Briefly, 1 μ g of total RNA was treated with Genomic DNA elimination mixture at 42 °C for 5 min and chilled on ice immediately for at least 1 min. Then, the RNA was reverse transcribed into first-strand cDNA using RT cocktail at 42 °C for 15 min. After reverse transcription, the reaction was heated at 95 °C for 5 min to degrade the RNA and to inactivate the reverse transcriptase. The resulting cDNA synthesis reaction was used for performing real-time PCR in RT2 qPCR master mix cocktail. All real-time RT-PCR assays were performed in duplicate in at least three independent experiments using two different tissue sets. The iCycler software was used to calculate threshold cycles. Relative expression value of each target gene was normalized to β -actin or Gapdh gene expression.

ChIP assay for Elf5 occupancy

Mouse mammary glands (fourth and fifth) at pregnancy day 17.5 were excised in aseptic conditions and minced in PBS. The tissue

Table 1
Primer sequences used in RT-PCR analysis.

Gene	Primer	Product size (bp)
β -Actin	Sense: ACCAACTGGGACGATATGGAGAAGA Antisense: TACGACCAGAGGCATACAGGACAA	214
β -Casein	Sense: GGTGAATCTCATGGGACAGC Antisense: CACAGGGGTTGAGCAATAG	160
α -Casein	Sense: CAGCATAGCAGCAGTGAGGA Antisense: CTTCTGAGCACTTGCCATT	172
Gapdh	Sense: CATGGCCTTCCTGTCTCTTA Antisense: CCTGCTTACCACCTCTTGAT	~200
Cldn7	Sense: AGCATGTTCTCTGGATTGGTC Antisense: CCAGAAGGACCAGAGCAGAC	128
Elf5	Sense: GTGGCATCAAGAGTCAAGACTGTC Antisense: CTCAGCTTCTCGTACGTCATCTG	220
HPRT	Sense: GCTGGTGAAGGACCTCT Antisense: CACAGGACTAGAACCTGCG	246
Id2	Sense: CTATCGTCAGCCTGCATCAC Antisense: GGAATTCAGATGCTCGA A	111
IKK α	Sense: CGTTAGCACAAATGATAGAAGA Antisense: TCATTCTGCTAACCAACTCCAA	125
K18	Sense: AAGGTCTGGAAGCCCAGATT Antisense: CTTGGTGTGACAACTGTGG	191
Lmo4	Sense: CCCAAGGCAACGTGTATCAT Antisense: GGGCTGTGGTCTATCATGT	126
Muc-1	Sense: TTCCAACCCAGGACACCTAC Antisense: ATTACCTGCCGAAACCTCCT	119
NKCC1	Sense: GGTTCCTCCAACTCAGCG Antisense: GTCTTGCCATCTCTTCTCTC	600
SOC3	Sense: GAGATTTTCGCTTCGGGACTA Antisense: ACTCGCTTTTGAGCTGAAG	200
SOC2	Sense: CGACTAACCTGCGGATTGAGTA Antisense: TGTCCGTTATCTCTTGACA	80
Stat5a	Sense: GTGAAGCCACAGATCAAGCA Antisense: GTCAAACCTGCCATCTTGTT	174
Wap	Sense: TATCATCTGCCAAACCAACG Antisense: GGTCGTGGAGCATTTCTATC	185

fragments were resuspended in 4 ml of PBS and crosslinked for 5 min at RT by adding formaldehyde (Sigma-Aldrich, St. Louis, MO) to a final concentration of 1%. The crosslinking reaction was stopped by adding glycine (Sigma-Aldrich, St. Louis, MO) to a final concentration of 125 mM and incubating for 5 min at room temperature. The tissue fragments were recovered by centrifugation for 10 min at 3000 \times g. Then, they were rinsed thrice with 8 ml of cold PBS and the tissue fragments were resuspended in cold cell lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 3 mM MgCl₂, 0.5% NP-40) containing 1 \times of Protease Arrest (G-Biosciences, St. Louis, MO) and transferred to a cold Dounce homogenizer (Rubins et al., 2005). After complete homogenization of the tissue, the nuclei were pelleted by centrifugation for 5 min at 12,000 \times g. Nuclei were lysed in 750 μ l of ChIP lysis buffer (ChIP kit, Upstate, Charlottesville, VA) and sonicated 16 times with 15-second cycles at setting 3 (Branson Sonifier 250) to generate fragments of 0.5 kb on average. Sonicated samples were centrifuged at 12,000 \times g for 10 min and the supernatant was transferred to a new tube. Aliquots of 180 μ l were diluted 10-fold in ChIP dilution buffer (EZ-ChIP kit, Millipore, Temecula, CA) then, pre-cleared by incubating with 75 μ l of 50% protein G-Sepharose beads slurry containing salmon sperm DNA and BSA (EZ-ChIP kit, Millipore, Temecula, CA) at 4 °C for 30 min. The pre-cleared chromatin was divided in aliquots of 600 μ l and they were immunoprecipitated overnight at 4 °C with 10 μ g of anti-Elf5 (N-20) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or normal goat IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or no antibody as control. The immunoprecipitation, washing, elution, reversing crosslink and proteinase K treatment were performed according to the manufacturer's directions described in the EZ-ChIP kit (Millipore, Temecula, CA). After proteinase K treatment, the immunoprecipitated DNA was purified with PCR purification kit (QIAGEN, Inc. Valencia, CA). PCR analysis for Elf5 occupancy in the proximal promoter of Stat5a was performed in a volume of 25 μ l containing 1 \times PCR buffer (Sigma-Aldrich, St. Louis, MO), 200 μ M dNTPs (Sigma-Aldrich, St. Louis, MO), 2 ng/ μ l of each P1 (CAGGAATCG-GAATGGAAAG) and P2 (GGAGCTTTCCTGGATCATA) primers, P3 (GAGCTGACACCCTCAAGGTC) and P4 (CTGGAGGGATTTTGAGCAAG) primers, 1.25 U Jumpstart Taq DNA polymerase (Sigma-Aldrich, St. Louis, MO) and 5 μ l template. The PCRs were carried out 95 °C for 4 min, followed by 35 cycles of 95 °C for 45 s, 58 °C for 45 s and 72 °C for 45 s, with a final extension step at the end of 72 °C for 5 min. The PCR amplification products were analyzed by gel electrophoresis in a 1.5% agarose gel in 1 \times TBE buffer at 120 V for 50 min and visualized with ethidium bromide staining and pictures were taken.

Results

Conditional deletion of exon 6 leads to functional inactivation of Elf5

Since the early embryonic lethality of the conventional knockout precluded studying the role of Elf5 in the development of adult epithelial cells, we employed the Cre-loxP recombination system to generate conditional Elf5 knockout mice. In these animals containing the floxed allele (hereafter referred to as Elf5^{f/f} mice), the loxP sites flank exon 6, which encodes for most of the Ets domain of the Elf5 gene (Fig. 1A and Fig. S1A, available as supplemental material). The deletion of the Ets domain is very likely to disrupt the critical biological functions of Elf5 protein since the Ets domain is the DNA binding domain necessary for transcriptional activity of Elf5 (Sharrocks, 2001). As shown by Southern blot analyses, the knockout strategy led to the expected rearrangements in the Elf5 genomic locus (Fig. S1B). Elf5^{f/f} mice developed normally and were phenotypically indistinguishable from their wild-type littermates. Since the Elf5^{f/f} animals harbored a phosphoglycerate kinase-neomycin (PGK-neo) cassette we also mated the Elf5^{f/f} animals with flipper (FLPe) mice to remove the PGK-neo cassette and one FLPe recognition target (FRT) site, leaving behind two loxP sites flanking exon 6 (Fig. S1A). The FLPe-mediated excision of the

PGK-neo cassette was verified using Southern blot analysis (Fig. S1C). Mice having two deleted alleles generated by the FLPe recombinase displayed no overt phenotypic abnormalities, indicating that the introduction of a PGK-neo cassette and two FRT sites had no adverse effect on the transcriptional regulation of Elf5.

To investigate the role of Elf5 in the mammary gland epithelium, the Elf5^{f/f} mice were bred first with the well-characterized mouse mammary tumor virus (MMTV)-Cre transgenic mice, which express Cre in luminal epithelium of the mammary gland (Wagner et al., 2001; Wagner et al., 1997). Although MMTV-Cre/Elf5^{+/f} animals were born in normal Mendelian ratios, very few MMTV-Cre/Elf5^{f/f} mice survived. However, in the animals that survived, targeted deletion of Elf5 by the MMTV-Cre transgene resulted in severe lactation defects in MMTV-Cre/Elf5^{f/f} females. Histological analysis showed poorly developed mammary epithelial structures in the few MMTV-Cre/Elf5^{f/f} females that were examined (Fig. S2). To overcome the lethality associated with MMTV-Cre/Elf5^{f/f} animals we crossed the Elf5^{f/f} animals with K14-Cre transgenic animals. We first examined the efficiency and specificity of K14-Cre-mediated recombination in mammary gland by crossing K14-Cre transgenic mice with ROSA26-LacZ reporter mice, and the LacZ (β -galactosidase) staining was observed in both luminal and myoepithelial cells (Fig. 1B). This observation is consistent with the fact that K14 promoter is active in a common progenitor cell type, which can commit to either luminal or myoepithelial cells (Dunbar et al., 2001; Woodward et al., 2005).

In contrast to MMTV-Cre/Elf5^{f/f} animals, K14-Cre/Elf5^{f/f} mice containing two floxed Elf5 alleles and K14-Cre transgene were obtained at the expected frequency from all crosses, viable and fertile. All mice of K14-Cre/Elf5^{+/+}, K14-Cre/Elf5^{+/f} and K14-Cre/Elf5^{f/f} reached puberty with no obvious phenotypic abnormalities. Examination of the skin samples by PCR showed that the Cre-mediated recombination events indeed took place as expected with the deletion of exon 6 in K14-Cre/Elf5^{+/f} and K14-Cre/Elf5^{f/f} animals (Fig. 1C). To confirm Elf5 deletion in mammary gland tissue, the level of Elf5 protein in the mammary gland was measured by Western blot analysis. As shown in Fig. 1D, Elf5 protein expression was significantly reduced in K14-Cre/Elf5^{+/f} mammary gland and completely lost in K14-Cre/Elf5^{f/f} mammary gland tissues at parturition. The absence of Elf5 protein in K14-Cre/Elf5^{f/f} mammary glands indicates that the targeting event has successfully generated a null mutation of Elf5 in mammary gland tissue. Interestingly, though Elf5 is highly expressed in skin epithelium, primarily in the hair follicles, K14-Cre-mediated deletion of Elf5 resulted in no apparent skin phenotype. However, both K14-Cre/Elf5^{+/f} and K14-Cre/Elf5^{f/f} females displayed dramatic defects in mammary gland development during pregnancy and lactation. The K14-Cre-mediated Elf5 mutant dams gave birth to pups whose size and numbers were normal. However, all pups suckling on the Elf5 mutant mothers, regardless of their genotype, died within a couple of days of birth. Examination of the pups revealed no milk in the stomach suggesting an inability of the Elf5 mutant mothers to lactate or nurse.

Elf5 is indispensable for mammary lobuloalveolar development

The fact that primiparous Elf5-deficient dams are unable to feed their pups suggested defects in alveologenesis and lactogenesis. To evaluate this we harvested mammary glands on the day after delivery of pups from wild-type, K14-Cre/Elf5^{+/f} and K14-Cre/Elf5^{f/f} females. Differentiation status of Elf5-deficient mammary tissue at parturition was strikingly different from that of wild-type counterpart, as judged by whole-mount and histological analyses (Fig. 2). During terminal differentiation, wild-type mature mammary ductal tree is decorated with well-differentiated lobuloalveolar units consisting of fully expanded alveoli filled with milk (Figs. 2A–C). However, a fairly low density of lobuloalveoli-like structures was observed in K14-Cre/Elf5^{+/f} mammary tissue, suggestive of defective

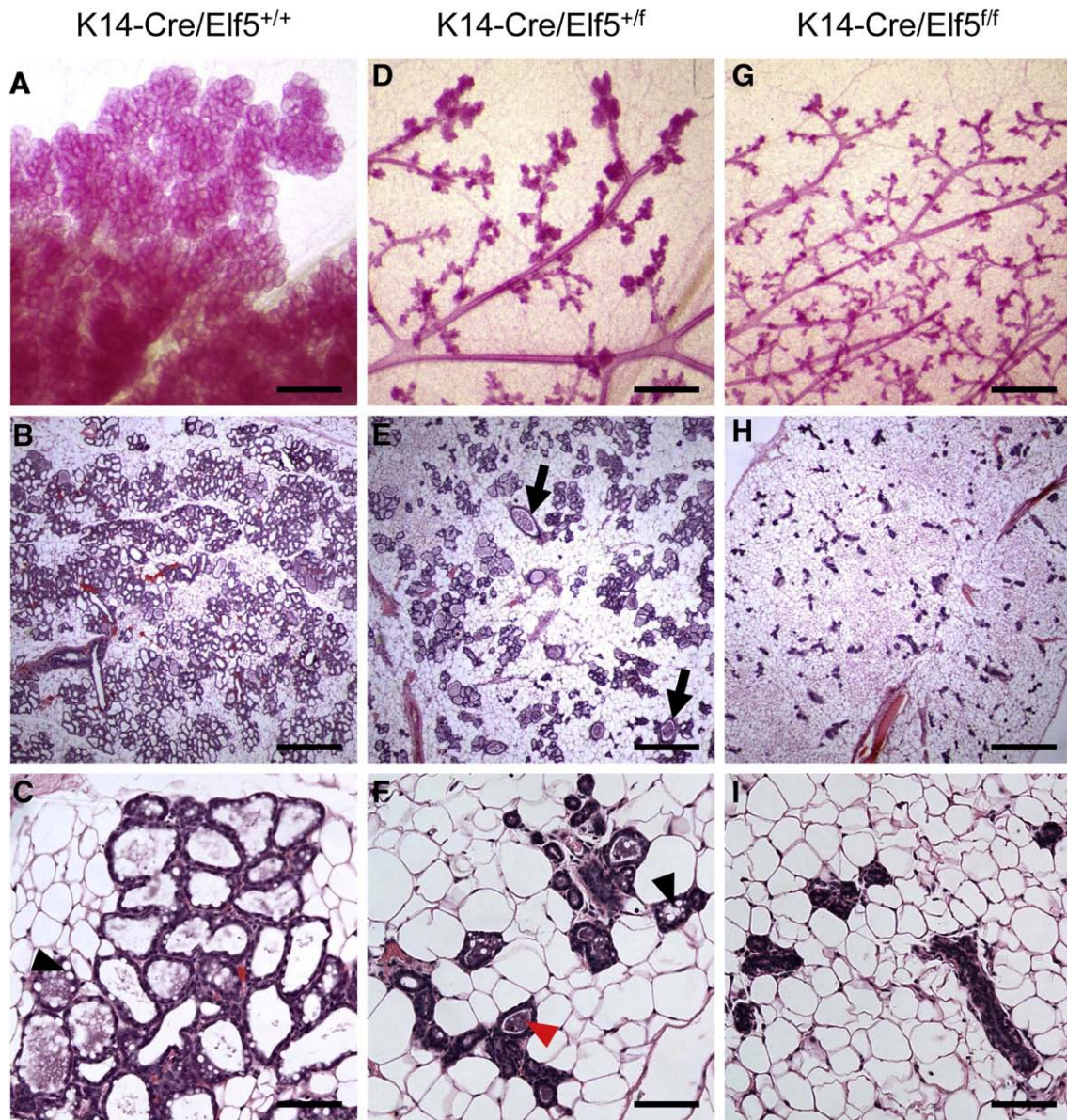


Fig. 2. Severely impaired lobuloalveolar development in *Elf5*-deficient females. (A–C) *K14-Cre/Elf5*^{+/+}; (D–F) *K14-Cre/Elf5*^{+/f}; (G–I) *K14-Cre/Elf5*^{f/f} mammary glands from primiparous dams on the day after delivery of pups. (A, D and G) Whole-mount analysis of mammary glands by carmine alum staining. (B, C, E, F, H and I) Histological analysis of mammary gland tissue sections by hematoxylin and eosin staining. Dilated ducts are indicated by the arrows in E. Black arrowheads indicate the presence of cytoplasmic lipid droplets (C and F). Red arrowhead denotes accumulated luminal secretory materials (F). Note that *Elf5*-null mammary tissues exhibit closed lumina and no lobuloalveoli (I). A representative specimen is shown as example in each case; the experiments were performed five times independently and yielded similar results ($n = 5$ for each genotype). Bar in A, B, D, E, G and H, 0.5 mm. Bar in C, F and I, 100 μ m.

differentiation (Figs. 2D–F). In addition, only minority of lumina displayed features of secretory differentiation with lipid droplets (Fig. 2F, black arrowhead), whereas most alveolar lumina were filled with condensed luminal secretory materials (Fig. 2F, red arrowhead). Accumulation of luminal secretory materials normally appears during midpregnancy in undifferentiated alveolar epithelium. Therefore, the retention of luminal secretory materials in *K14-Cre/Elf5*^{+/f} mammary alveoli at parturition further implies that the *K14-Cre/Elf5*^{+/f} mammary epithelial cells fail to undergo terminal differentiation. Histological analysis also showed large lobuloalveolar units composed of many individual alveoli that were lined by flattened, vacuolated epithelial cells in wild-type mammary tissue sections (Fig. 2C). In contrast, the *K14-Cre/Elf5*^{+/f} mammary tissue was still mostly composed of adipose stroma and the parenchyma consisted of dilated ducts (Fig. 2E, black arrow) and small clusters of alveoli-like structures that were lined by packed cuboidal epithelial cells (Fig. 2F). While the histological features of *K14-Cre/Elf5*^{+/f} mammary tissue at parturition

were partially reminiscent of immature alveoli of wild-type, the defects in the mammary epithelial cells in *K14-Cre/Elf5*^{f/f} mice were more pronounced (Figs. 2G–I). Notably, whole-mount and histological analyses revealed that *K14-Cre/Elf5*^{f/f} mammary tissue did not have the appearance of lobuloalveoli-like structures and were completely underdeveloped. While in postpartum mammary tissue of wild-type mice, adipose tissue was replaced by lobuloalveolar structures, the *K14-Cre/Elf5*^{f/f} mammary tissue was largely composed of adipocytes and its fat pad was almost entirely filled with ducts. Interestingly, the majority of *K14-Cre/Elf5*^{f/f} epithelial architecture at parturition consistently exhibited closed lumen (Fig. 2I). Interestingly, this defect in *K14-Cre/Elf5*^{f/f} mothers was consistently exhibited even after multiple pregnancies, indicating that the complete loss of *Elf5* could not be compensated by multiple exposures to pregnancy (Fig. 3A). Unlike *K14-Cre/Elf5*^{f/f} dams, primiparous *K14-Cre/Elf5*^{+/f} females consistently failed to lactate but multiparous *K14-Cre/Elf5*^{+/f} females often were able to lactate. As shown in Fig. 3B, the partial

loss of Elf5 in the K14-Cre/Elf5^{+/-} mammary glands resulted in immature lobuloalveoli at the first pregnancy, while the lactating mammary glands, which were restored after multiple pregnancies displayed fully expanded alveoli containing lipid droplets. The successful lactation after numerous pregnancies could be partially explained by a cell population that might have survived through involution (Briskin et al., 1999).

Elf5-null mammary epithelium at parturition retains characteristics of virgin ductal epithelial cells

To identify the epithelial cells lacking Elf5 at parturition as ductal or alveolar, we investigated the expression of specific markers. First, Elf5-deficient mammary glands were evaluated by immunofluorescence staining of ductal markers, AQP5 and Na–K–Cl cotransporter 1 (NKCC1). Normally, NKCC1 and AQP5 are present at high levels in the ductal epithelial cells of virgin mammary gland and there is a diminution of NKCC1 and AQP5 levels during pregnancy and at parturition (Shillingford et al., 2002; Shillingford et al., 2003). Our immunostaining studies showed that AQP5 and NKCC1 expression persisted in postpartum Elf5-deficient mammary epithelia (white arrowhead in Figs. 4D, E, G, H), while it was undetected in lactating wild-type mammary tissue (Figs. 4A and 4B). The retention of significantly high levels of NKCC1 mRNA in Elf5-deficient mammary glands at parturition further supports this observation (data not shown).

The Na–Pi cotransporter isoform, Npt2b is expressed on the apical membrane of secretory alveolar epithelium in lactating mammary gland and serves as a marker of secretory function (Shillingford et al., 2002; Shillingford et al., 2003). The absence of Npt2b protein at parturition is indicative of a lack of secretory function (Miyoshi et al., 2001). As expected, the apical membranes of secretory alveoli in wild-type mammary glands stained intensely for Npt2b (Fig. 4C). The alveoli in K14-Cre/Elf5^{+/-} mammary glands displayed a mosaic staining pattern of Npt2b at parturition with expression evident only on the apical membrane of a few secretory cells (Fig. 4F, white arrowhead) and was absent from most alveoli-like structures (Fig. 4F, yellow arrowhead). In contrast, apical Npt2b expression was not at all detectable in K14-Cre/Elf5^{-/-} mammary tissues at parturition, indicating a critical defect in terminal epithelial differentiation (Fig. 4I). Interestingly, in the Elf5 knockout mammary gland, apart for the usual basolateral surfaces, NKCC1 staining also was observed in the apical surface of some population (20–30%) of epithelial cells implying that Elf5-deficient luminal epithelial cells fail to properly polarize (Figs. S3 A–C, yellow arrowhead). This defect in cell polarity is further evident with the aberrant staining pattern for Laminin, a basal marker, which shows distinct localization of laminin in the basolateral sides of luminal epithelium of Elf5-deficient mammary glands at parturition (Figs. S3 D–F, yellow arrowhead) similar to those observed in MMTV-β1-integrin transgenic mice (Faraldo et al., 1998). We hypothesize that this defect in the cellular polarity might be the partial underlying cause for the closed lumen structures observed in the K14-Cre/Elf5^{+/-} mammary glands.

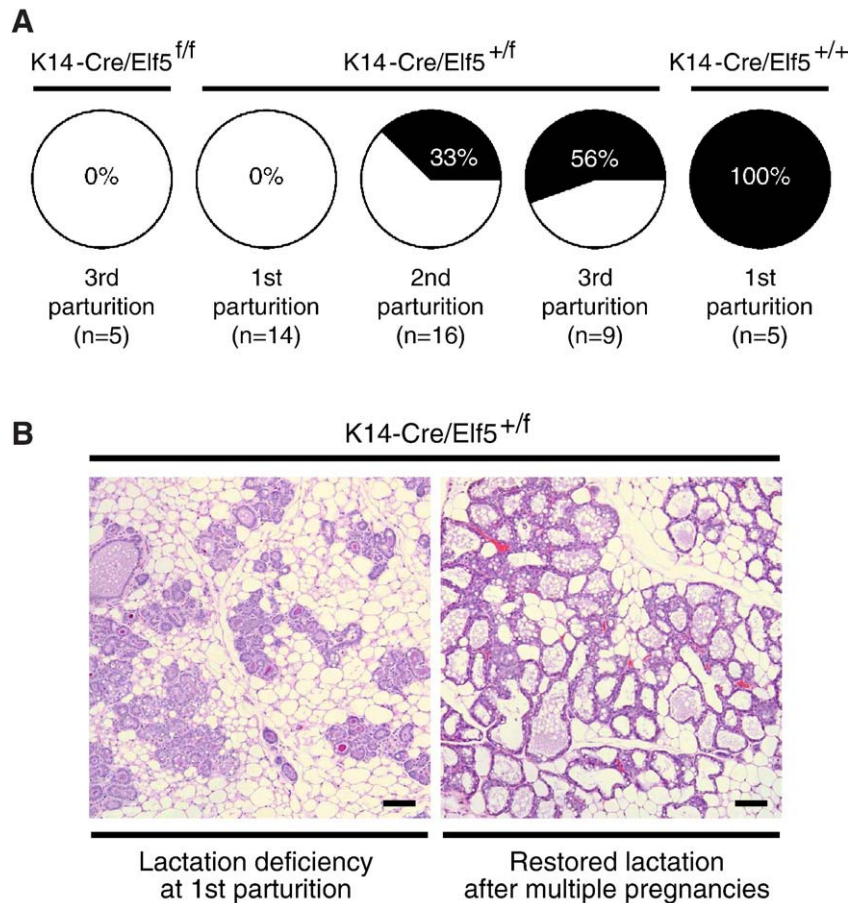


Fig. 3. Restoration of lactation in K14-Cre/Elf5^{+/-} dams after multiple pregnancies. (A) Percentage of animals capable of lactation was determined from triparous K14-Cre/Elf5^{-/-} (0%; n=5), uniparous K14-Cre/Elf5^{+/-} (0%; n=14), biparous K14-Cre/Elf5^{+/-} (33%; n=16), triparous K14-Cre/Elf5^{+/-} (56%; n=9) and uniparous K14-Cre/Elf5^{+/+} (100%; n=5) females. Black color space in a pie chart represents the proportion of the total number of females capable of lactation. Note that all uniparous and biparous K14-Cre/Elf5^{-/-} females were unable to lactate. (B) Histological analysis of lactating mammary tissue in K14-Cre/Elf5^{+/-} females. Mammary gland sections from the first pregnant (left panel) and the restored biparous (right panel) females on the day after delivery of pups were stained with hematoxylin and eosin. At parturition, primiparous K14-Cre/Elf5^{+/-} females show impaired mammary alveoli, while restored multiparous K14-Cre/Elf5^{+/-} females have fully expanded alveoli containing lipid droplets. Bar, 100 μm.

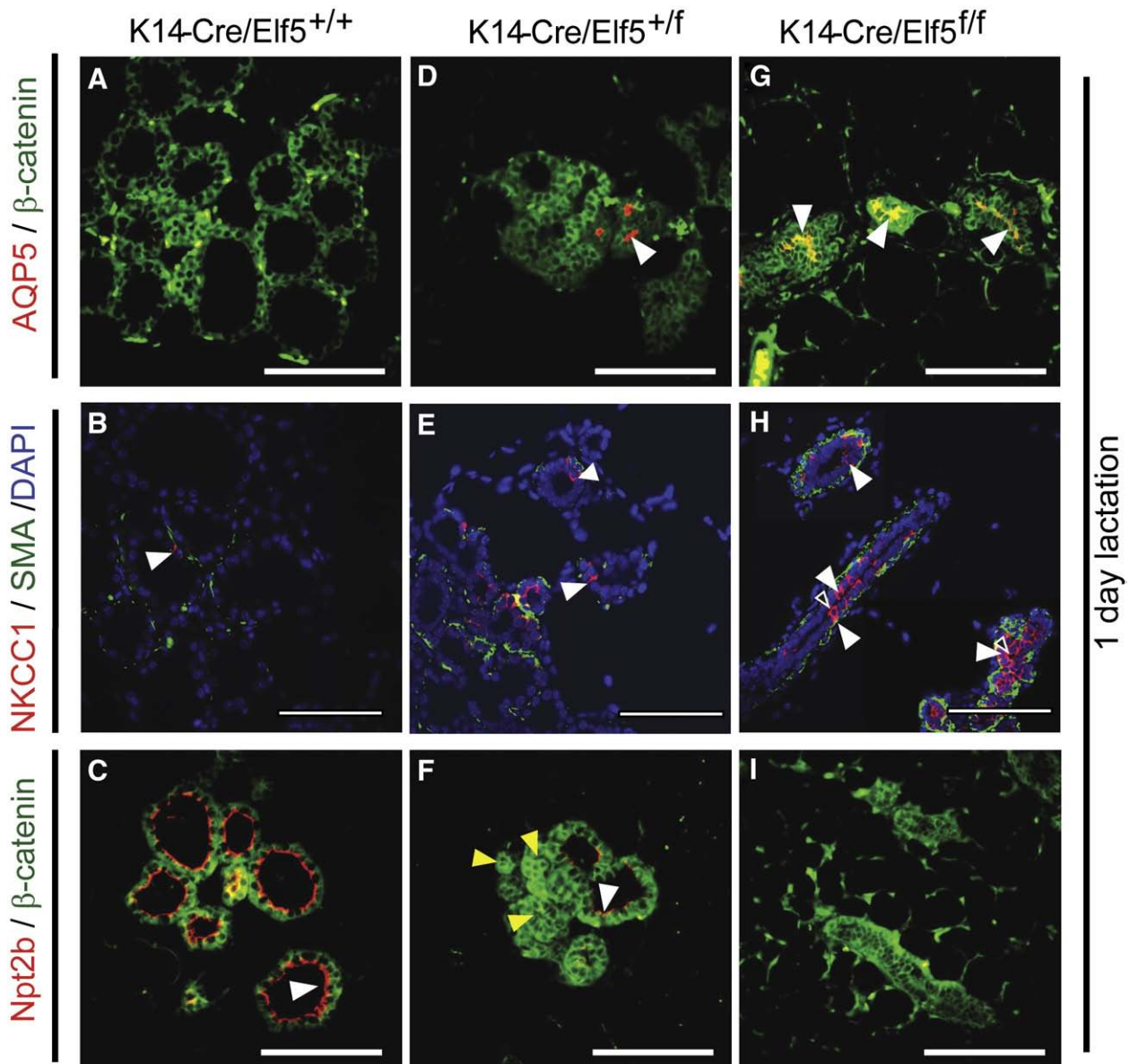


Fig. 4. Failure of alveolar differentiation in K14-Cre/Elf5^{f/f} mammary gland. All mammary glands were harvested from females on the day after delivery of pups. Immunofluorescence staining of AQP5 (red; A, D, G), NKCC1 (red; B, E, H) and Npt2b (red; C, F, I). β -Catenin (green) was normally expressed in the basolateral membrane of tissue sections examined, and was used to visualize cell membranes (A, C, D, F, G, I). SMA (smooth muscle actin, green) was used to label the myoepithelial cells (B, E, H). White arrowheads in D and G and E and H indicate retained expression of AQP5 and NKCC1 in the Elf5-deficient mammary epithelium in comparison to the wild-type (A and B). Some occasional cells show staining of NKCC1 in the wild-type mammary epithelium as indicated by a white arrowhead (B). White arrowhead in C indicates Npt2b staining of the apical surface of the wild-type secretory epithelium. Yellow arrowheads pointed to the absence of Npt2b expression in alveoli-like structures (F). Note no positive Npt2b staining in K14-Cre/Elf5^{f/f} mammary tissue (I). Bar, 100 μ m.

Taken together, the absence of Npt2b and the continued expression of NKCC1 and AQP5 in mammary glands lacking Elf5 suggested that the characteristic features of virgin ductal epithelial cells persisted in Elf5-null mammary epithelium at parturition, most likely due to failure of ductal epithelial cells to undergo cellular specification to secretory alveolar cells accompanied by alterations in cellular polarity and orientation.

Reduced proliferation of Elf5-null mammary epithelium associated with persistence of estrogen receptor alpha (ER α) and progesterone receptor (PR) positive cells

Proliferation of mammary alveolar epithelium occurs throughout pregnancy and during early postpartum period. We used Ki67 staining to determine whether defective alveolar development in Elf5-deficient mice was a result of impaired proliferation. When compared

to a K14-Cre/Elf5^{+/+} mammary gland at the same developmental time periods, a significant reduction in Ki67 positive epithelial cells was observed in K14-Cre/Elf5^{f/f} mammary tissue (Figs. 5A, D) suggesting that reduced proliferation might partly contribute to the defects in lobuloalveolar development along with defective differentiation of secretory cells.

Cell proliferation in the mammary gland is under the control of multiple hormones and their effectors such as the ER α and PR. While luminal cells of a virgin mammary gland and early pregnancy exhibit an abundant presence of ER α and PR, during late pregnancy and lactation stages expression of these receptors is downregulated (Figs. 5B, C). To test if the failure of alveolar cells to proliferate was also associated with aberrant hormone response state we stained K14-Cre/Elf5^{f/f} mammary tissue for ER α and PR. As expected, expression of ER α and PR was retained in the Elf5-deficient luminal cells during lactation in accordance with the reduced proliferation state (Figs. 5E, F).

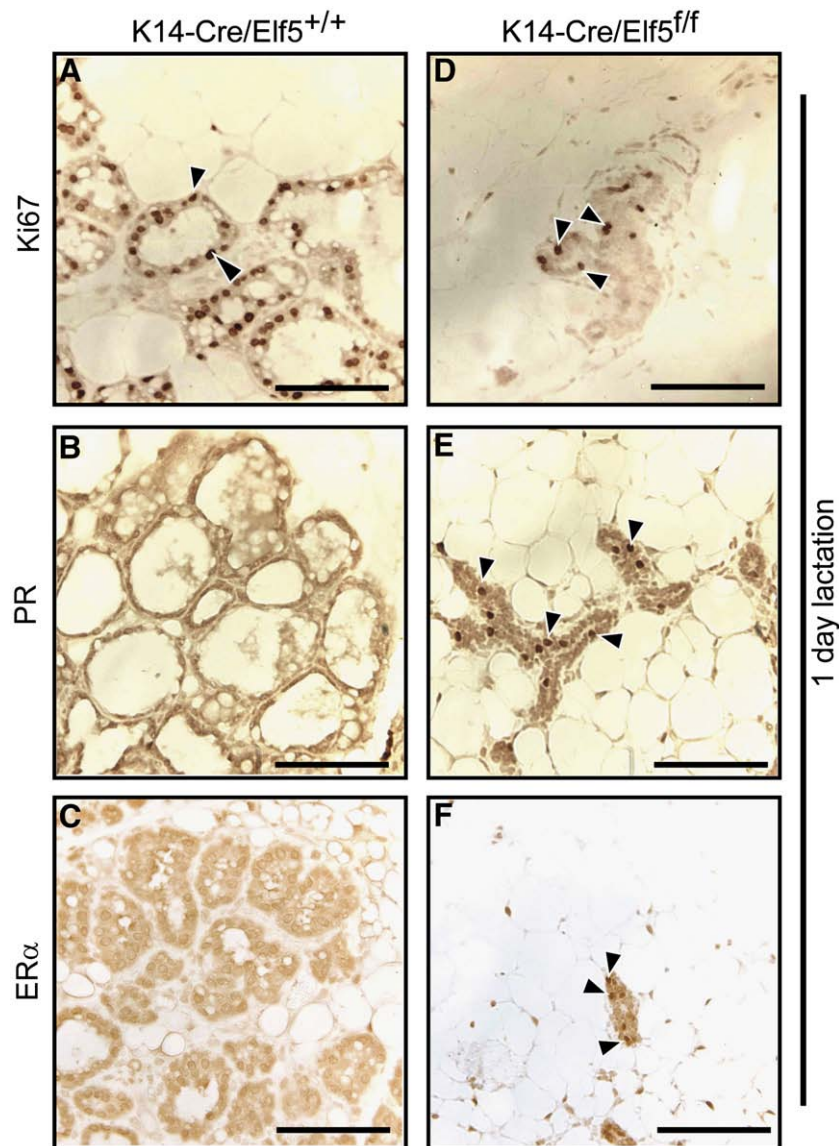


Fig. 5. K14-Cre/Elf5^{f/f} mice exhibit defects in mammary epithelial proliferation. Immunohistochemical detection of Ki67 in K14-Cre/Elf5^{+/+} mammary epithelium (A) and K14-Cre/Elf5^{f/f} mammary epithelium (D). A significant reduction in the percentage of Ki67 positive cells was observed in K14-Cre/Elf5^{f/f} mammary epithelium. Immunohistochemical staining with PR and ERα show retained expression of PR and ERα in K14-Cre/Elf5^{f/f} epithelium (E and F) compared to wild-type mammary epithelium (B and C). Bar, 40 μm.

Elf5-null mammary epithelium lacks activated Stat5 and fails to undergo functional differentiation

It has been previously shown that ectopic expression of Elf5 can rescue the impaired alveologenesis of mammary tissue lacking PrlR, implying that Elf5 would be involved in the prolactin-mediated mammary differentiation (Harris et al., 2006). The PrlR/Jak2/Stat5 signaling has been well known to regulate mammary gland development during pregnancy and lactation (Briskin and Rajaram, 2006; Hennighausen and Robinson, 2005; Miyoshi et al., 2001; Oakes et al., 2006). Immunohistochemistry of Elf5 in wild-type mammary tissue showed that the nuclei of most luminal cells were uniformly positive for Elf5 (Fig. 6A). However, the Elf5 in K14-Cre/Elf5^{+/f} mammary tissue was expressed in a mosaic pattern (Fig. 6E) and complete ablation of Elf5 expression was confirmed in K14-Cre/Elf5^{f/f} mammary epithelium (Fig. 6I). To determine whether alterations in PrlR/Jak2/Stat5 signaling pathway are responsible for the failed lobuloalveolar development in Elf5-null mice, we first analyzed the expression levels of PrlR, Janus kinase 2 (Jak2) and signal transducer and activator of transcription 5 (Stat5), which are involved in tyrosine phosphor-

ylation and activation of Stat5 protein. Quantitative RT-PCR analyses indicated that while PrlR and Jak2 levels were unchanged, Stat5a levels were decreased in Elf5-deficient mammary glands compared with wild-type counterparts (Fig. S4). The reduced expression of Stat5a in Elf5-deficient mammary tissue was further verified in terms of protein expression by immunofluorescence staining of Stat5a. While a strong signal for Stat5a was detected in most alveolar cells of wild-type mammary epithelia (Fig. 6B), fewer Stat5a-positive cells were detected in Elf5-mutant epithelia (Figs. 6F, J). In addition, a monoclonal antibody, which is specific to tyrosine (Y694) phosphorylated Stat5 (p-Stat5), was used to detect the activated Stat5 proteins. As expected, prominent nuclear staining of p-Stat5 was uniformly observed in mammary alveolar cells of wild-type mice at parturition (Fig. 6C). However, K14-Cre/Elf5^{+/f} mammary tissue at parturition displayed a mosaic pattern of p-Stat5 expression (Fig. 6G), consistent with the Elf5 expression pattern observed in heterozygous Elf5 mutant mammary tissue. Strikingly, despite the normal expression of PrlR and Jak2, p-Stat5 was almost completely suppressed in the Elf5-null mammary epithelial cells at parturition (Fig. 6K). Although our analyses showed reduced Stat5a expression in Elf5-null mammary

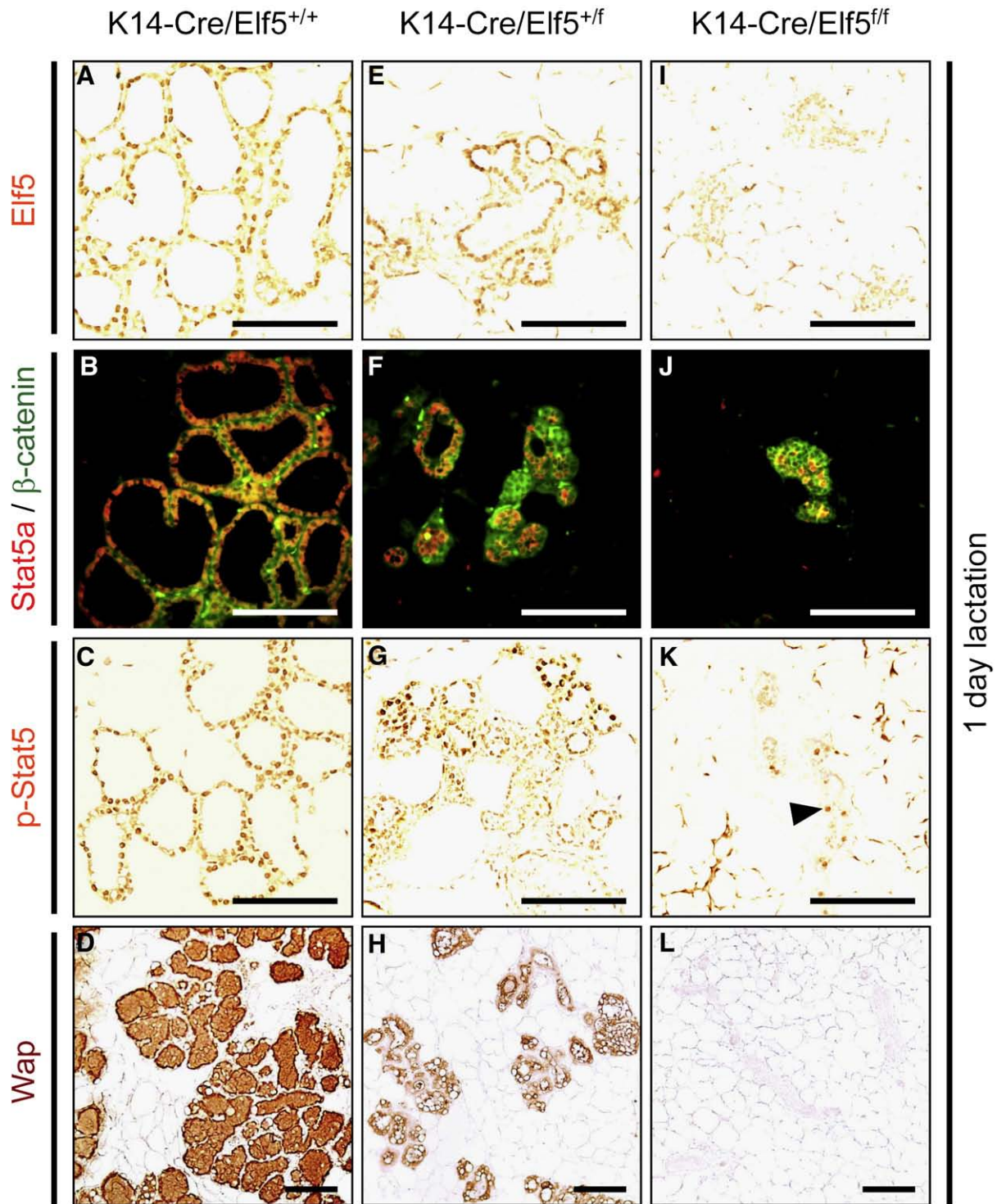


Fig. 6. Downregulation of p-Stat5 and the absence of Wap in Elf5-null mammary gland at parturition. All mammary tissues were harvested from females on the day after delivery of pups. (A–L) Immunostaining of Elf5, Stat5a, p-Stat5 and Wap in mammary tissues of primiparous K14-Cre/Elf5^{+/+} (A–D), K14-Cre/Elf5^{+/f} (E–H) and K14-Cre/Elf5^{f/f} (I–L). Sections were stained with anti-Elf5 (A, E and I), anti-Stat5a (red; B, F and J), anti-β-catenin (green; B, F and J), anti-p-Stat5 (C, G and K) or anti-Wap (D, H and L) antibodies. Positive staining for Elf5 or p-Stat5 is noted in the nuclei of luminal cells and Wap-positive staining is shown in the lumina and the luminal border. Note that Elf5-null mammary tissues display no positive Elf5 (I) and Wap (L) staining and very few positive staining with Stat5a (J) and p-Stat5 (K, arrowhead). A representative specimen is shown as an example in each case; the experiments were performed three times independently and yielded similar results ($n = 3$ for each genotype). Bar, 100 μ m.

epithelia at parturition, Elf5 is likely to contribute partially to the regulation of *Stat5a* gene expression in mammary epithelial cells since considerable levels of Stat5a were still maintained in Elf5-null epithelia. It also does appear that the extent of p-Stat5 down-regulation was more than what could be explained by that reduced levels of Stat5a suggesting that additional pathways involved in Stat5

phosphorylation may be compromised. We also examined the phosphorylation status of Stat5 in the mammary glands of K14-Cre/Elf5^{f/f} at postpuberty (12 week virgin), midpregnancy (P12.5) and late pregnancy (P17.5). Immunostaining indicated reduced staining of p-Stat5 that was pronounced in P12.5 and P17.5 samples of K14-Cre/Elf5^{f/f} mammary glands (Fig. S5). Western blot analysis on enriched

mammary epithelial cell lysates of K14-Cre/Elf5^{f/f} at P17.5 confirmed the reduced expression of p-Stat5 compared to the K14-Cre/Elf5^{+/+} samples (data not shown). Taken together, these data raise an interesting possibility that Elf5 can not only modulate the PrlR/Jak2-mediated activation of Stat5 but also can regulate Stat5 transcription in mammary epithelial cells.

To examine the functional differentiation status in Elf5-null mammary epithelium, we determined expression of whey acidic protein (Wap) which is a major milk protein in mice. Wap is known to be regulated by Elf5 and/or Stat5 transcription factors since the binding sites for Elf5 or Stat5 are found within Wap promoter regions (Derbinski et al., 2008; Rosen et al., 1999; Thomas et al., 2000). Immunohistochemistry of Wap showed a striking difference in milk production between Elf5 mutant and wild-type mammary glands. As predicted, the Wap protein was highly abundant in secretory alveoli of lactating wild-type mammary glands (Fig. 6D). In contrast, weak expression of Wap was observed in K14-Cre/Elf5^{+/f} mammary tissue (Fig. 6H) and the Elf5-null mammary epithelia lacking p-Stat5 completely failed to express Wap protein at parturition (Fig. 6L). These data were confirmed by Western blot analysis of Wap expression (data not shown). These results clearly demonstrate that mammary epithelial cells do not undergo functional differentiation/lactogenesis in the absence of Elf5 and that the absence of activated Stat5 may contribute to this phenotype.

Loss of Elf5 leads to transcriptional downregulation of genes involved in alveolar development

In an effort to gain further insight into the molecular mechanisms underlying the Elf5-mediated alveolar development, we sought to examine potential target genes of Elf5. For this purpose we focused our attention to two distinct groups of molecules: ones that are part of the broader Jak/Stat pathway and others that have been implicated in alveolar homeostasis based on genetic and/or biochemical evidence. Quantitative RT-PCR analyses of the mammary glands lacking Elf5 (either homozygous or heterozygous) revealed that expression of many of such genes involved in alveolar development were altered while compared with the wild-type mammary glands. Changes in selected transcripts are shown in Fig. 7. Unlike the reduced Stat5a expression levels in the K14-Cre/Elf5^{f/f} mammary glands, mRNA expression levels of other Stat family members were comparable to

those of wild-type counterparts (data not shown). We also examined the Suppressor of cytokine signaling (SOCS) family, since SOCS members negatively regulate the PrlR/Jak2-mediated activation of Stat5 (Starr et al., 1997; Tomic et al., 1999). Interestingly expression of two such SOCS genes, SOCS2 and SOCS3 was significantly upregulated in mammary tissue lacking Elf5 (Fig. 7).

Quantitative RT-PCR analysis showed that the Id2 (inhibitor of DNA binding 2), IKK α (inhibitor of kappa B kinase alpha) and Lmo4 (LIM domain only 4) mRNA expression were all downregulated three- to four-fold in postpartum Elf5-null mammary gland (Fig. 7). Interestingly, these genes are known to play important roles in alveolar and lactogenic differentiation as demonstrated by mouse knockout studies (Cao et al., 2001; Desprez et al., 2003; Miyoshi et al., 2002; Mori et al., 2000; Sum et al., 2005). Other significant changes in mRNA expression levels were observed in cytokeratin 18 (K18), mucin 1 (Muc-1) and claudin 7 (Cldn7). The intermediate filament protein K18, transmembrane glycoprotein Muc-1 and membrane-associated protein Cldn7 are expressed in mammary luminal epithelial cells (Blackman et al., 2005; Hilken et al., 1992; Mikaelian et al., 2006) and their reduced expression might reflect the reduction in the epithelial compartment in Elf5 null mammary gland (Fig. 7). It has been shown that Elf5 can transcriptionally upregulate K18 expression in lung epithelial cells by interacting with putative Elf5 binding sites located in the first intron of K18 (Yaniw and Hu, 2005); we speculate that this mechanism may also be operative in mammary epithelial cells. In addition, Cldn7 has been reported to be reduced in PrlR-deficient mammary tissue (Ormandy et al., 2003), its reduction in Elf5 knockout animals most likely reflects the fact that Elf5 is an important downstream component of the PrlR signaling pathway. On the contrary, by quantitative RT-PCR, we found no change in expression of β -catenin (Fig. 7), an essential factor for alveolar cell survival (Tepera et al., 2003), in agreement with immunofluorescence staining with anti- β -catenin antibodies in Elf5 null mammary tissue. The wide range of critical genes altered by the loss of Elf5 suggests that these could be potential targets of Elf5 in mammary gland development.

Elf5 is required for Stat5a expression and activation in cultured mammary epithelial cells

To directly examine the role of Elf5 in regulating Prl signaling and Stat5 activity and in governing the alveolar differentiation program we

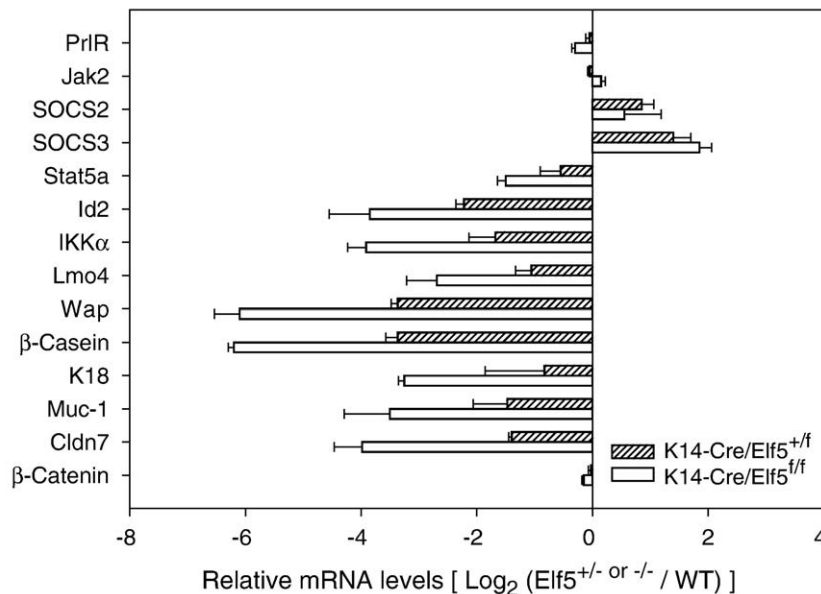


Fig. 7. Elf5-mediated gene regulation in mammary alveolar development. Gene expression analysis of mammary glands from K14-Cre/Elf5^{+/+}, K14-Cre/Elf5^{+/f} or K14-Cre/Elf5^{f/f} mice at parturition. The real-time RT-PCR assays were performed in at least three independent experiments using two different tissue sets. Data are presented as the mean \pm SD.

assessed the effects of the loss of Elf5 in primary mammary epithelial cells (MECs). MECs were harvested from $\text{Elf5}^{\text{f/f}}$ mice and infected with either Ad-Cre or Ad-GFP and cultured on BM matrix for varying time points. Infection of MECs with adenovirus expressing Cre resulted in significant knockdown of Elf5 expression by 48 h after infection (data not shown) with virtually no detectable protein after 72 h (Figs. 8A and B). Interestingly, Ad-Cre-infected cells showed no apparent abnormal morphological phenotype and formed mammary organoids similar in size and appearance to the control $\text{Elf5}^{\text{f/f}}$ cells (Fig. S6) when observed after 72 h. To examine Stat5 activity, MECs were grown for 24 h in growth media and then cultures were exposed to differentiation media containing prolactin for another 48 h. Protein lysates were harvested from differentiated MECs and Western blots were performed. Concomitant with the loss of Elf5 in Ad-Cre-infected $\text{Elf5}^{\text{f/f}}$ MECs, there was a significant reduction in the expression of Casein, suggesting that Elf5 null cells fail to differentiate. Similarly, there was a modest decrease in the overall levels of Stat5a, but a dramatic reduction in the levels of p-Stat5 (Fig. 8B). This data corroborates our in vivo findings from K14-Cre/ $\text{Elf5}^{\text{f/f}}$ mice and suggest that the acute loss of Elf5 not only leads to decreased expression of Stat5a but also causes an even more profound effect on Stat5 activity by regulating its phosphorylation status.

Having established the Elf5-null MEC system, we next investigated whether the loss of expression of some of the critical mediators described in Fig. 7 in the Elf5-null mammary glands is due to a direct transcriptional event mediated by Elf5 or merely indicative of the block in differentiation phenotype. We performed Quantitative RT-PCR analyses of the primary cultures lacking Elf5 (Ad-Cre- $\text{Elf5}^{\text{f/f}}$) to examine the expression levels of some of the genes as shown in Fig. 8C. Interestingly both Stat5a and Wap transcripts were significantly downregulated in Ad-Cre- $\text{Elf5}^{\text{f/f}}$ compared to (Ad-GFP- $\text{Elf5}^{\text{f/f}}$) cells. In agreement with the data obtained from Elf5-null mammary glands, expression of SOCS2 and SOCS3 was significantly upregulated in Elf5-deleted MECs. However, some of the transcripts such as Id2 and Cldn7 did not show any significant change suggesting that these might reflect indirect effects of the loss of Elf5 in mammary glands.

Stat5 is a direct transcriptional target of Elf5

Having established Stat5 as a potential downstream target of Elf5, we wondered if Elf5 directly regulates the expression of Stat5. To address this, we searched for Elf5-response elements (Elf5RE) in the proximal promoter region of Stat5 based on sequence similarities to the Elf5 consensus site as defined by our laboratory (Choi and Sinha,

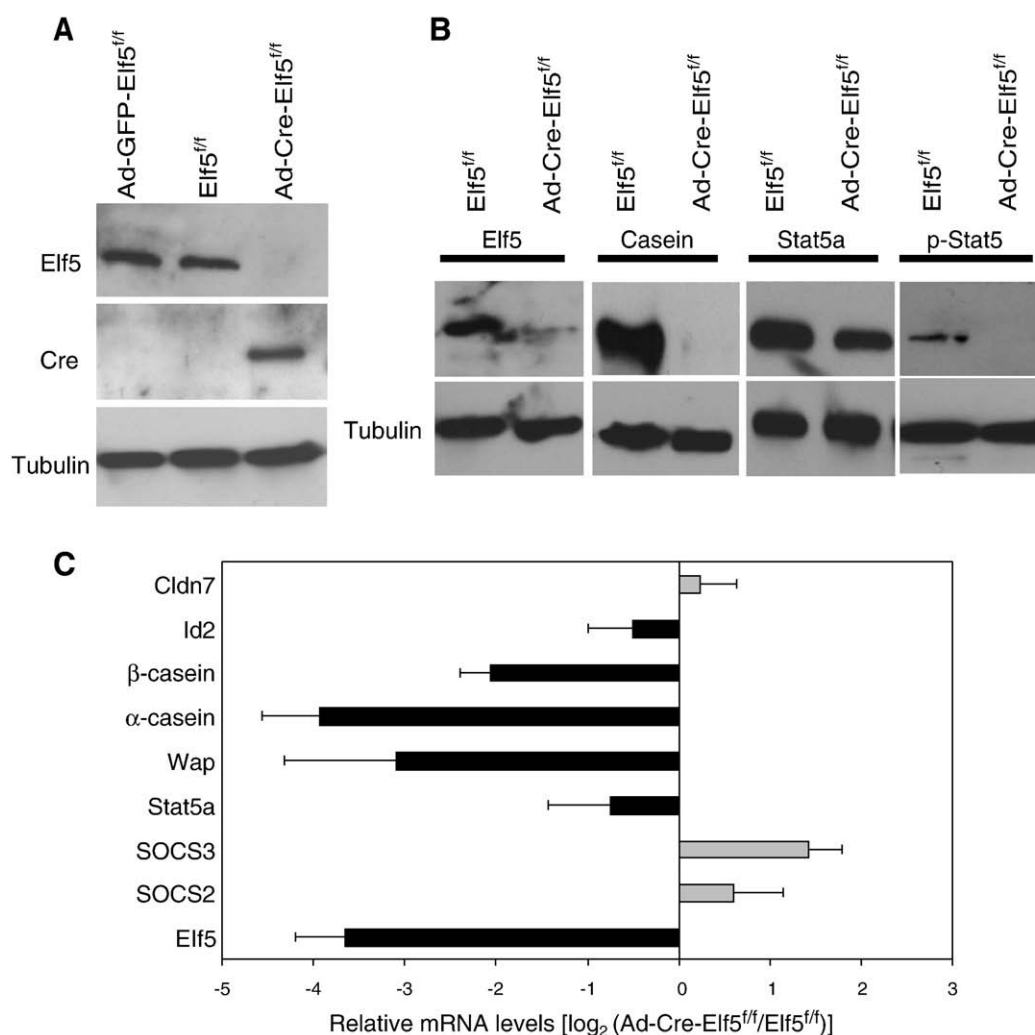


Fig. 8. Elf5 regulates prolactin-mediated differentiation and Stat5 activity. (A) $\text{Elf5}^{\text{f/f}}$ primary mammary epithelial cells (MECs) were infected with Ad-GFP or Ad-Cre in suspension and plated on BM matrix. Western blot analysis of protein lysates from $\text{Elf5}^{\text{f/f}}$ MECs infected with Ad-Cre resulted in knockdown of Elf5 in differentiation medium containing prolactin (A) and (B). (B) The loss of Elf5 also blocked differentiation as demonstrated by decreased levels of Casein, Stat5a and p-Stat5. (C) Gene expression analysis of MECs from control and $\text{Elf5}^{\text{f/f}}$ mice infected with Ad-Cre at 72 h. The real-time RT-PCR assays were performed in duplicates using three independently generated samples. In the absence of Elf5, milk proteins like Wap, α casein and β casein are down regulated. Loss of Elf5 also led to reduced expression of Stat5a mRNA and up-regulation of SOCS2 and SOCS3 transcripts. Changes in Id2 and Cldn7 levels were not statistically significant.

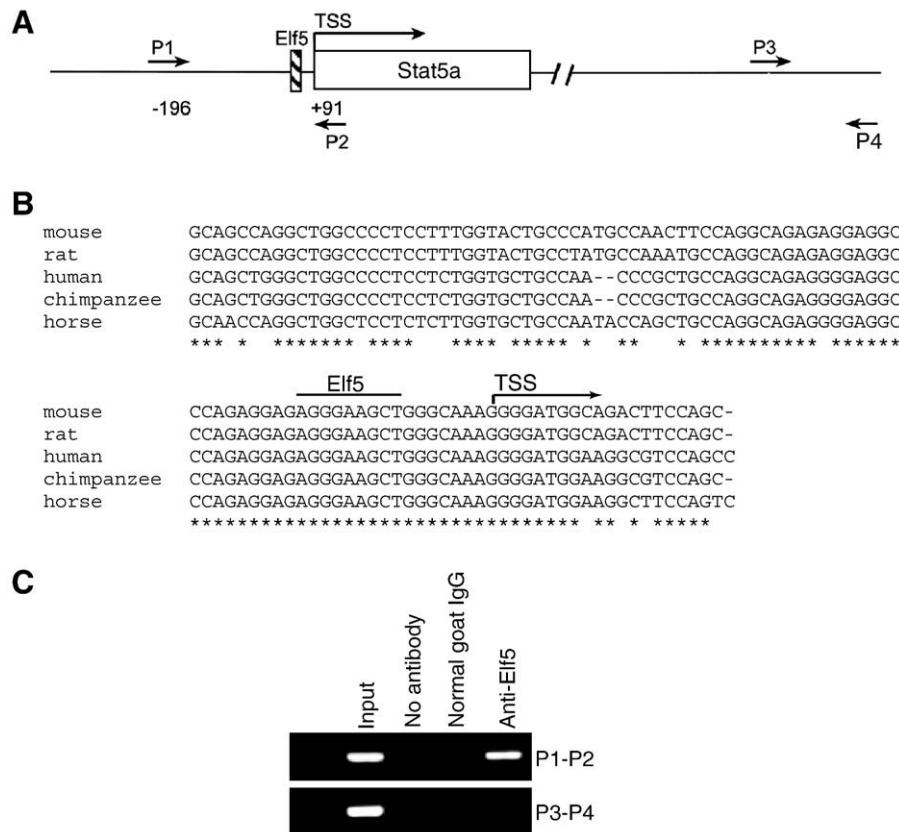


Fig. 9. ChIP analysis for Elf5 occupancy in the proximal promoter of Stat5a in mouse mammary gland. (A) Schematic diagram of the proximal promoter of mouse Stat5a gene showing the location of the primers (P1 and P2) that were used to amplify the region that contains a putative Elf5 binding site. A region 3' of the Stat5 gene was also amplified using primers P3 and P4 as a negative control. The location of the transcription start site (TSS) is also indicated. (B) Sequence fragments of Stat5a proximal promoter from various species were obtained from Ensemble genome database and aligned using the ClustalW2 software. The * sign indicated conserved residues. The line shows the putative Elf5 binding sites (with a GGAA core) and the arrow indicates the putative TSS. (C) Elf5 occupancy by ChIP analysis using the primer combination P1/P2 and P3/P4 in whole tissue of mouse mammary gland (pregnancy day 17.5) with anti-Elf5 and as well as with normal goat IgG or without any antibody as indicated. The PCR amplification of the input represents 1% of the chromatin DNA used for the ChIP. Results are representative of three independent experiments.

2006). Although several potential Elf5RE were identified, one specific element close to the transcriptional start site of the mouse Stat5 gene showed the highest level of sequence similarity between several species (Figs. 9A and 9B). The evolutionarily conserved nature of the Elf5RE suggested the possibility of a functionally important role for this DNA element. We next verified whether Elf5 was able to interact with the promoter of the Stat5 gene *in vivo*, by performing ChIP (Chromatin immunoprecipitation) assays in mammary gland. For this purpose we utilized a specific antibody against Elf5, that has been shown to be effective in ChIP experiments as well as a control IgG antibodies (Ng et al., 2008). As shown in Fig. 9C, our PCR data clearly shows a specific amplification of the Stat5 proximal promoter region but not of a randomly selected genomic segment 3' to the Stat5 gene in Elf5-immunoprecipitated genomic DNA compared to the control IgG antibodies. This suggests that Elf5 can directly interact with regulatory regions of the Stat5 in mammary epithelium.

Discussion

To identify essential functions of Elf5 during mammary gland development, we have generated the first mammary gland-specific Elf5 knockout mice utilizing the Cre-lox system. While some phenotypic consequences of Elf5 deficiency in mammary glands have been reported previously, embryonic lethality associated with the complete loss of Elf5 have limited these studies to Elf5 heterozygotes or mammary transplants. These studies have established that Elf5 plays an important role in lobuloalveolar development and milk secretion and serves as a critical mediator of the prolactin

hormonal pathway. Our results described in this report extend these prior observations and provide new mechanistic insights into the function of Elf5 as an essential modulator of PrIR/Jak2-mediated activation of Stat5 and a master regulator in alveolar and lactogenic development.

In agreement with prior studies, we document that the critical role of Elf5 is during alveologenesis since in its absence, there is a complete block in this stage of mammary gland development. Thus Elf5-null mammary epithelium at parturition retains characteristics of virgin ductal epithelial cells with persistence of marker proteins NKCC1 and AQP5 and a failure of induction of Np2tb. In glandular epithelia such as the mammary gland, establishing proper basoapical polarity axis such that the basal surface of the cells is against the extracellular basement membrane, and the apical surface is next to the lumen, is of paramount importance in maintaining functional differentiation. In the absence of Elf5, we find that a subpopulation of luminal cells exhibit an aberrant polarity—with an altered localization of surface marker proteins such as Laminin and NKCC1. Furthermore, we also observe reduced proliferation of Elf5-null mammary epithelium associated with persistence of estrogen receptor alpha (ER α) and progesterone receptor (PR) positive cells, a phenomenon also observed in other knockout models associated with blocked lobuloalveolar development (Grimm et al., 2002). The reduced proliferative potential of Elf5 null cells along with their altered cellular orientation may significantly contribute to the block in lobuloalveolar development along with defective differentiation of secretory cells.

Not surprisingly, the severely defective alveoli-like structures observed in K14-Cre/Elf5^{f/f} mammary glands are reminiscent of

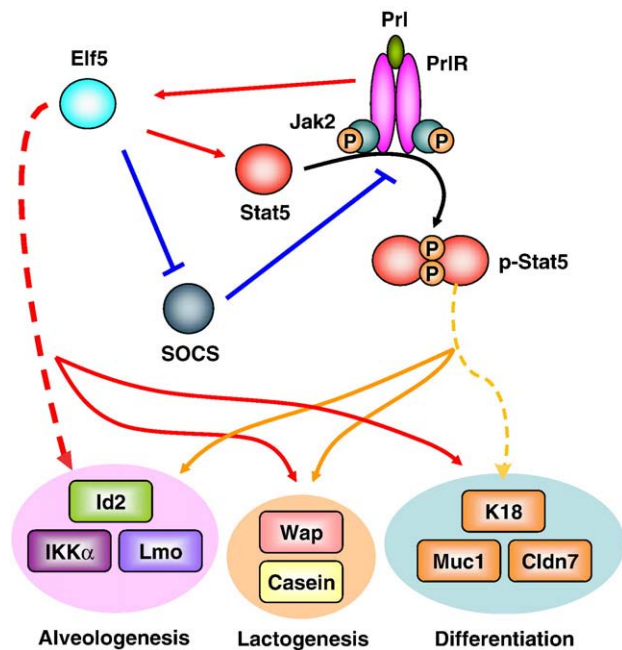


Fig. 10. Prospective model explaining the effect of Elf5 on alveolar and lactogenic differentiation. In the absence of Elf5, Stat5 expression is reduced and SOCS2 and SOCS3 expression are upregulated. These events lead to downregulation of PrlR/Jak2-mediated activation of Stat5. Activatory event is noted with a red or orange line terminating in an arrowhead, and the inhibitory event is marked with a blue line ending with a bar. The solid line indicates genes affected by the direct loss of Elf5 and the dashed line indicates genes for which the effects are likely to be indirect.

phenotypes in PrlR- and Stat5-null mammary glands (Miyoshi et al., 2001). The phenotypic overlap between these knockout mouse models implies that Elf5 transcription factor is directly or indirectly associated with PrlR/Jak2/Stat5 signaling pathway. Interestingly, unlike the Stat5a or PrlR-knockouts, but similar to Jak2 conditional knockouts, multiple gestation cycles did not reverse this phenotype, suggesting that other factors are not able to compensate for the loss of Elf5 function. Interestingly, although the decrease of the overall Stat5a levels induced by Elf5 loss was quite modest, we observed a drastic decrease of p-Stat5 expression. One possibility is that Elf5 may modulate the PrlR/Jak2-mediated phosphorylation of Stat5 through regulating activities of genes involved in tyrosine phosphorylation of Stat5 such as PrlR and Jak2. However, the expression of both PrlR and Jak2 was not altered by Elf5 deletion. This result prompted us to examine another possible modulation of phosphorylation of Stat5 by Elf5-regulated inhibitors of the PrlR/Jak2 signaling since negative regulators of the PrlR/Jak2/Stat5 signaling pathway such as SOCS family members suppress the biological activities of the PrlR and Jak2 proteins without transcriptional downregulation (Pezet et al., 1999; Tomic et al., 1999). Interestingly, multiple members of the SOCS family were upregulated by the loss of Elf5. This suggests a dual mechanism by which the loss of Elf5 leads to a significant attenuation of the PrlR/Jak2/Stat signaling pathway by both directly inhibiting Stat5 expression levels and concurrently upregulating negative mediators of Stat5 phosphorylation, thus leading to the loss of activated p-Stat5. This is quite in agreement with our recent studies suggesting that Elf5 can function as both transcriptional activator and repressor (Choi and Sinha, 2006). However, it needs to be stressed that although Elf5 is one of the critical regulator of the PrlR-mediated activation/phosphorylation of Stat5, parallel pathways such as those mediated by ERBB4 also operate during pregnancy induced functional differentiation of mammary epithelium and may have overlapping roles (Long et al., 2003).

It has been hypothesized that Elf5 is a downstream target of Stat5 (Oakes et al., 2006). Although, we do not rule out this possibility, our

data seem to indicate that Elf5 is more likely to be upstream mediator of the PrlR/Jak2/Stat pathway. As shown by ChIP experiments, we find Elf5 binding site in the proximal Stat5 promoter that is occupied by Elf5. This correlates well with our observation that in Elf5-null mammary glands and MECs, there is downregulation of Stat5 expression. The fact that Elf5 expression is not significantly altered in the Stat5 null animals (Dr. L. Hennighausen, personal communication) is further evidence in support of this argument. Finally, our studies suggest that the effects of the loss of Elf5 are not limited to the expression of critical members of PrlR/Jak2/Stat pathway but also many other important critical players of lobuloalveolar development (see Model in Fig. 10). At this stage, it is difficult to discern whether the loss of expression of all of these critical mediators in the Elf5-null mammary glands is a reflection as direct transcriptional targets of Elf5 or merely indicative of the block in differentiation phenotype. Our preliminary data with Elf5-null MECs seems to indicate that Elf5 can potentially directly regulate the expression of many of the target genes important for lobuloalveologenesis, while for others, its effect may be more indirect. Future studies to uncover the transcriptional targets of Elf5 will be very useful in shedding light on the molecular mechanisms by which this important transcription factor controls several aspects of mammary gland development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ydbio.2009.02.032](https://doi.org/10.1016/j.ydbio.2009.02.032).

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